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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/82, 15/56, 15/11 C12N 5/10, A01H 5/00

A1

(11) International Publication Number: WO 91/16440

(43) International Publication Date: 31 October 1991 (31.10.91)

(21) International Application Number: PCT/GB91/00614

(22) International Filing Date: 19 April 1991 (19.04.91)

(30) Priority data:
9009307.1 25 April 1990 (25.04.90) GB

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(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CM (OAPI patent), DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL, NL (European patent), NO, PL, RO, SD, SE, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent), US.

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: DNA, DNA CONSTRUCTS, CELLS AND PLANTS DERIVED THEREFROM

(57) Abstract

DNA constructs useful for modifying the ripening behaviour of fruit comprise a transcriptional initiation region operative in plants positioned for transcription of a DNA sequence homologous to some or all of a gene encoding cellulase or a like enzyme, said gene showing substantial homology to DNA encoded by either of the constructs TCELB6 and MCELE2. Also plant cells and plants transformed with such constructs.

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DNA, DNA CONSTRUCTS, CELLS AND PLANTS DERIVED THEREFROM

This application relates to novel DNA constructs, plant cells containing them and plants derived therefrom. In particular it involves the use of recombinant DNA technology to control gene expression in plants.

As is well known, a cell manufactures protein by transcribing the DNA of the gene for that protein to produce messenger RNA (mRNA), which is then processed (eg by the removal of introns) and finally translated by ribosomes into protein. This process may be inhibited by the presence in the cell of "antisense RNA". By this term is meant an RNA sequence which is complementary to a sequence of bases in the mRNA in question: complementary in the sense that each base (or the majority of bases) in the antisense sequence (read in the 3′ to 5′ sense) is capable of pairing with the corresponding base (G with C, A with U) in the mRNA sequence read in the 5′ to 3′ sense. It is believed that this inhibition takes place by formation of a complex between the two complementary strands of RNA, preventing the formation of protein. How this works is uncertain: the complex may interfere with further transcription, processing, transport or translation, or degrade the mRNA, or have more than one of these effects. Such antisense RNA may be produced in the cell by transformation with an appropriate DNA construct arranged to transcribe backwards part of the coding strand (as opposed to the template strand) of the relevant gene (or of a DNA sequence showing substantial homology therewith).

The use of this technology to downregulate the expression of specific plant genes has been described, in for example European Patent publication no 271988 to ICI (corresponding to US serial 119614). Reduction of gene expression has led to a change in the phenotype of the plant: either at the level of gross visible phenotypic difference e.g. lack of anthocyanin production in flower petals of petunia leading to colourless instead of coloured petals (van der Krol et al, Nature, 333, 866-869, 1988); or at a more subtle biochemical level e.g. change in the amount of polygalacturonase and reduction in depolymerisation of pectins during tomato fruit ripening (Smith et al, Nature, 334, 724-726, 1988; Smith et al., Plant Molecular Biology, 13, 303-311, 1990). Thus antisense RNA has been proven to be useful in achieving downregulation of gene expression in plants.

In work leading to the present invention we have isolated DNA sequences encoding cellulase or like enzymes from tomato and melon. We postulate that these will be of use in modifying the ripening characteristics of tomatoes, melons and other fruit.

The cell walls of tomato and melon fruit predominantly consist of polysaccharides which have been sub-divided into cellulose, hemicellulose and pectin fractions. During fruit ripening there are considerable changes in the composition of the cell walls. In tomato pericarp tissue the proportion of cellulose in cell wall fractions increases slightly (Huber, Horticultural Science 20, 442-443, 1985).

A similar slight increase in cellulose content has been observed in ripening melons. However since other cell wall fractions are rapidly

solubilised and degraded, the absolute level of cellulose may decline during ripening. In the tomato locular gel the proportion of cellulose in the cell wall fractions decreases during ripening.

Increases in cellulase activity have been correlated with avocado fruit ripening (Awad and Young, Plant Physiology 64, 306-308, 1979). The role of cellulase in tomato and melon fruit softening is unclear.

There have been several reports of cellulase activity in tomato fruit (eg Hall, Nature 200 1010-1011,1963 and Hobson, Journal of food science 33, 588-592, 1968). Cellulase activity has been detected in mature green tomato fruit at the onset of ripening. In pericarp tissue, cellulase activity increases approximately ten fold as ripening progresses (Poovaiah and Nukaya, Plant Physiology 64, 534-537, 1979 and Huber, 1985 cited above). Cellulase activity is higher in the locular gel than in the pericarp and also increases during ripening. It has been suggested that cellulase activity may particularly be an important feature of gel formation.

In ripening melons cellulase activity was reported to be highest in youngest tissues and then declined as fruit matured and ripened (Lester and Dunlap, Scientia Horticulturae 26, 323-331, 1985).

According to the present invention we provide DNA constructs comprising a transcriptional initiation region operative in plants positioned for transcription of a DNA sequence homologous to some or all of a gene encoding cellulase or a like enzyme, said gene showing substantial homology to DNA encoded by either of the constructs TCELB6 and MCELE2.

In a further aspect, the present invention provides such DNA constructs comprising a transcriptional initiation region operative in plants positioned for transcription of a DNA sequence encoding RNA complementary to a substantial run of bases showing substantial homology to an mRNA encoding cellulase or a like enzyme. The invention also includes plant cells containing constructs of the invention; transformed plants derived therefrom showing modified ripening characteristics; and fruit and seeds of such plants

The constructs of the invention may be inserted into plants to regulate the production of cellulase or like enzymes. Depending on the nature of the construct, the production of the enzyme may be increased, or reduced, either throughout or at particular stages in the life of the plant. Generally, as would be expected, production of the enzyme is enhanced only by constructs which contain DNA homologous to the substantially complete gene. What is more surprising is that constructs containing an incomplete DNA sequence substantially shorter than that corresponding to the complete gene generally inhibit the expression of the enzyme, whether they are arranged to express sense or antisense RNA.

The genes used in the invention typically derive from DNA from tomato or melon cellulase genes; or from DNA which is fully or partly homologous thereto. This invention may be put into effect using the clones TCELB6 or MCELE2 that have been deposited. Alternatively such clones may be used as probes to identify other homologous cellulase or like genes (or parts thereof) in plant DNA, thereby obtaining DNA in longer lengths or having variant sequences. It is possible to screen

in this way DNA derived from tomato or melon, or from other plant material known to obtain cellulase genes.

The plants to which the present invention can be applied include commercially important fruit-bearing plants, in particular tomato and melon. In this way, plants can be generated which express RNA from recombinant DNA and which may have one or more of the following characteristics:

Delayed softening and improved firmness;
Reduced spoilage of fruit during handling, due to delayed softening but continued development of colour, flavour and aroma during ripening on the plant (this should allow the fruit to be harvested closer to the ripe fruit stage but still withstand handling and transport to arrive at the market in good condition);
Longer shelf life and better storage characteristics due to reduced cell wall degradation (the fruit may also be less prone to infection in storage);
Improved processing characteristics due to changed activity of cellulase contributing to factors such as: viscosity of paste, solids content, pH, elasticity.

DNA constructs according to the invention preferably comprise a base sequence at least 20 bases in length for transcription into RNA. If enhancement of expression of the enzyme is the objective, then substantially the whole of the gene sequence should be included in the construct. For inhibition by sense RNA, a shorter sequence is used. Where antisense RNA is used for inhibition, there is no theoretical upper limit to the length of the base sequence - it may be as long as the

relevant mRNA produced by the cell - but for convenience it will generally be found suitable to use sequences of at least 50 and preferably between 100 and 1000 bases in length. The preparation of such constructs is described in more detail below.

As source of the DNA base sequence for transcription, it is convenient to use clones such as TCELB6 for tomato cellulase and MCELE2 for melon cellulase. The base sequences of TCELB6 and MCELE2 are set out in Figure 1. Searches in DNA and protein data bases indicate that these clones show homology to clones for avocado fruit cellulase (Tucker et al , Plant Molecular Biology 9, 197-203, 1987) and bean abscission cellulase (Tucker et al Plant Physiology 88, 1257-1262, 1988). TCELB6 and MCELE2 have been deposited on 29 March 1990 with the National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland under Accession Nos. NCIB 40268 and 40269, respectively.

DNA fragments similar to those cloned in TCELB6 and MCELE2 may be generated in polymerase chain reactions using appropriate synthetic oligonucleotide primers and either tomato or melon genomic DNA as appropriate. Alternatively, cDNA clones showing homology to TCELB6 and MCELE2 may be obtained from the mRNA of ripening tomatoes or melons by methods similar to those described by Slater et al, Plant Molecular Biology 5, 137-147, 1985. In this way may be obtained sequences coding for the whole, or substantially the whole, of the mRNA produced by tomato or melon cellulase genes. Suitable lengths of the cDNA so obtained may be cut out for use by means of restriction enzymes.

The source of DNA fragments for the base sequence for transcription may be derived from

either mRNA or from a gene encoding a cellulase or like enzyme. If the DNA is derived from a gene it may differ from that derived from mRNA in that introns may be present. The introns are not transcribed into mRNA (or, if so transcribed, are subsequently cut out). When using such a gene as the source of the base sequence for transcription it is possible to use either intron or exon regions.

A further way of obtaining a suitable DNA base sequence for transcription is to synthesise it *ab initio* from the appropriate bases, for example using Figure 1 as a guide. Devices such as the Applied Biosystems oligonucleotide synthesiser are available which will synthesise lengths of single stranded DNA in any desired base sequence up to a maximum of around 100 bases. Complementary strands of DNA are annealed and subsequently ligated to form a double stranded DNA fragment of the desired length and sequence.

In the new vectors expressing antisense RNA, the strand that was formerly the template strand becomes the coding strand, and *vice versa*. The new vector will thus encode RNA in a base sequence which is complementary to the sequence of the cellulase gene. Thus the two RNA strands are complementary not only in their base sequence but also in their orientations (5′ to 3′).

Recombinant DNA and vectors according to the present invention may be made as follows. A suitable DNA source containing the desired base sequence for transcription (for example TCELB6 or MCELE2) is treated with restriction enzymes to cut the sequence out. Alternatively a suitable fragment may be generated by polymerase chain reaction (PCR) from a suitable DNA source (for

example TCELB6 or MCELE2) using synthetic oligonucleotide primers. The DNA strand so obtained is cloned (if desired in reverse orientation) into a second vector containing the desired promoter sequence (for example cauliflower mosaic virus 35S RNA promoter or the tomato polygalacturonase gene promoter sequence - Bird et al., Plant Molecular Biology, 11, 651-662, 1988) and the desired terminator sequence (for example the 3' of the Agrobacterium tumefaciens nopaline synthase gene, the nos 3' end).

According to the invention we propose to use both constitutive promoters (such as cauliflower mosaic virus 35S) and inducible or developmentally regulated promoters (such as the ripe-fruit-specific polygalacturonase gene) as circumstances require. Use of a constitutive promoter will tend to affect functions in all parts of the plant: while by using a tissue-specific promoter, functions may be controlled more selectively. Thus in applying the invention, e.g. to tomatoes and melons, it may be found convenient to use the promoter of the PG gene (Bird et al, 1988, cited above). Use of this promoter, at least in tomatoes, has the advantage that the production of antisense RNA is under the control of a ripening-specific promoter. Thus the antisense RNA is only produced in the organ in which its action is required. Other tomato ripening-specific promoters that could be used include the E8 promoter (Diekman & Fischer, EMBO Journal 7, 3315-3320, 1988).

Vectors according to the invention may be used to transform plants as desired, to make plants according to the invention. Dicotyledonous plants, such as tomato and melon, may be transformed by

Agrobacterium Ti plasmid technology, for example as described by Bevan (1984) Nucleic Acid Research, 12, 8711-8721. Such transformed plants may be reproduced sexually, or by cell or tissue culture.

The degree of production of antisense RNA in the plant cells can be controlled by suitable choice of promoter sequences, or by selecting the number of copies, or the site of integration, of the DNA sequences according to the invention that are introduced into the plant genome. In this way it may be possible to modify ripening or senescence to a greater or lesser extent.

The constructs of our invention may be used to transform cells of both monocotyledonous and dicotyledonous plants in various ways known to the art. In many cases such plant cells (particularly when they are cells of dicotyledonous plants) may be cultured to regenerate whole plants which subsequently reproduce to give successive generations of genetically modified plants. Examples of genetically modified plants according to the present invention include, as well as tomatoes, fruits of such as mangoes, peaches, apples, pears, strawberries, bananas and melons. Such genetically modified plants may further contain other exogenous DNA expressible under the control of plant promoters, for example DNA expressing RNA antisense to other fruit ripening enzymes, for example polygalacturonase or pectin methylesterase, as described in our European Patent application 271,988 (US Serial 119,614).

The invention will now be further described with reference to the drawings, in which:

Figure 1 shows the base sequences of tomato and melon cellulase genes in clones TCELB6 (Seq ID No: 1) and MCELE2 (Seq ID No: 2) respectively.

Figure 2 shows the oligonucleotides used for generation of fragments of tomato and melon cellulase gene by polymerase chain reaction.

Figure 3 shows a strategy for generation of fragments of a tomato cellulase gene by polymerase chain reaction.

Figure 4 shows the base sequence of tomato cellulase cDNA clone lambda cel-1 (Seq ID No: 3)

Figure 5 shows a strategy for construction of a cellulase antisense RNA vector pJR1TC1 according to the invention.

The following Examples illustrate aspects of the invention:

EXAMPLE 1

Synthesis, cloning and characterisation of a fragment of a tomato cellulase gene.

A fragment of a tomato cellulase gene was generated in a polymerase chain reaction. Synthetic oligonucleotide primers for the reaction (TCEL10 and TCEL11) were designed from regions of homology between avocado (Tucker et al, 1987, cited above) and bean abscision (Tucker et al, 1988, cited above) cellulase (Figure 2). In a PCR reaction with avocado DNA, these primers would be expected to generate a 266 base pair fragment based on the sequence in Tucker et al, 1987. PCR reactions (Figure 3) with DNA extracted from tomato

(Lycopersicon esculentum Mill cv. Ailsa Craig) using appropriate conditions for annealing and extension generated a fragment of approximately 300 base pairs. This fragment was purified from an agarose gel and cloned into the vector M13mp18 cut with HincII. 21 clones were transferred to microtitre plates and replicated on to nylon membranes (Hybond N - Amersham). The membranes were hybridised with [32P] labelled oligonucleotide TCEL10 in 5xSSPE, 0.25% Marvel, 0.05% SDS at 45oC and washed with 2xSSC, 0.1% SDS at 50oC. Seven clones hybridised to TCEL10. The nucleotide sequence of one of the hybridising clones (TCELB6) was determined (Figure 1). This had significant similarity to the nucleotide sequence of the avocado cellulase gene (Tucker et al, 1987, cited above).

EXAMPLE 2

Synthesis, cloning and characterisation of a fragment of a melon cellulase gene.

A fragment of a melon cellulase gene was generated, cloned and sequenced in a manner similar to that described in example 1 for the tomato cellulase gene fragment. The same PCR primers (TCEL10 and TCEL11) were used to generate a fragment of approximately 300 base pairs from DNA extracted from melon (Cucumis melo L cv. Western Shipper). This fragment was cloned into M13mp18 cut with HincII and hybridised with [32P] labelled TCEL10. The nucleotide sequence of one hybridising clone (MCELE2) was determined (Figure 1) and had significant homology to the tomato and avocado cellulase genes.

EXAMPLE 3

Isolation and characterisation of a tomato cellulase cDNA clone.

The insert of the clone TCELB6 was used as a hybridisation probe to screen a commercially available ripe tomato (Lycopersicon esculentum Mill cv Ailsa Craig) cDNA library. One hybridising clone (lambda cel-1) was identified and purified to homogeneity. The nucleotide sequence of the clone was completely determined (figure 4). The clone had an insert of 1415 base pairs and showed significant similarity to the avocado sequence (Tucker et al cited above).

EXAMPLE 4

Construction of tomato and melon cellulase antisense RNA vectors with the gene fragments amplified by PCR and the CaMV 35S promoter.

Vectors may be constructed using cloned sequences from tomato or melon cellulase gene or cDNA fragments as shown in Figure 5.

1. TCELB6 (223bp tomato cellulase gene) - pJR1TC1

2. MCELE2 (223bp melon cellulase gene) - pJR1MC1

pJR1TC1 may be synthesised in vitro by cutting TCELB6 RF DNA with PstI the cut ends are then made flush with T4 DNA polymerase. The DNA is then cut with BamHI. The 283bp fragment from this reaction is then isolated and cloned into pJR1 cut with SmaI and BamHI. pJR1 (Smith et al Nature 334, 724-726, 1988) is a Bin19 (Bevan, Nucleic Acids Research, 12, 8711-8721, 1984) based vector, which

permits the expression of the antisense RNA under the control of the CaMV 35S promoter. This vector includes a nopaline synthase (nos) 3′ end termination sequence.

After synthesis, vectors with the correct structure of pJR1TC1 are identified by DNA sequence analysis.

The vector pJR1MC1 is made similarly, following the construction schemes shown in Figure 5.

EXAMPLE 5

Construction of tomato and melon cellulase antisense RNA vectors with the gene fragments isolated by PCR and the tomato polygalacturonase gene promoter.

The tomato and melon gene fragments described in example 3 are also cloned by the same procedure into pJR2 to give the following clones:

1. TCELB6 (223bp tomato cellulase gene) - pJR2TC1

2. MCELE2 (223bp melon cellulase gene) - pJR2MC1

pJR2 is a Bin19 based vector, which permits the expression of the antisense RNA under the control of the tomato polygalacturonase promoter. This vector includes a nopaline synthase (nos) 3′ end termination sequence (see Figure 4).

EXAMPLE 6

Construction of tomato and melon cellulase sense RNA vectors with the gene fragments isolated by PCR and the CaMV 35S promoter.

The tomato and melon gene fragments described in example 3 are also cloned into pJR1 in the sense orientation to give the following clones:

1. TCELB6 (223bp tomato cellulase gene) - pJR1TC1S

4. MCELE2 (223bp melon cellulase gene) - pJR1MC1S

pJR1TC1S may be synthesised in vitro by cutting TCELB6 RF DNA with PstI and XbaI, the cut ends are then made flush with T4 DNA polymerase. The 283bp fragment from this reaction is then isolated and cloned into the HincII site of pJR1. After synthesis, vectors with the sense orientation of TCELB6 sequence are identified by DNA sequence analysis. The vector pJR1MC1S is made similarly.

EXAMPLE 7

Construction of tomato antisense and sense RNA vectors with the tomato cellulase cDNA insert.

The tomato cellulase cDNA clone (lambda cel-1) was cut with EcoRI to excise the cDNA insert. The 1415 base pair fragment was isolated and the cut ends were made flush with T4 DNA polymerase. This fragment was then cloned into the SmaI site of pJR1. After synthesis, vectors with the antisense orientation of the lambda cel-1 sequence were identified by both PCR and DNA sequence analysis. One clone that contained the cellulase cDNA sequence in the antisense orientation was designated pJRTCelA.

A vector pJRTCelS may be obtained by using a similar strategy and identifying a clone with the cellulase cDNA sequence in the sense orientation.

EXAMPLE 8

Generation of transformed tomato and melon plants

Vectors made as described in Examples 4-7 are transferred to Agrobacterium tumefaciens LBA4404 (a

micro-organism widely available to plant biotechnologists) and are used to transform tomato and melon plants. Transformation of tomato stem segments follows standard protocols (e.g. Bird et al Plant Molecular Biology 11, 651-662, 1988). Melon plants are transformed by a similar process. Transformed plants are identified by their ability to grow on media containing the antibiotic kanamycin. Plants are regenerated and grown to maturity. Ripening fruit are analysed for modifications to their ripening characteristics.

We claim:

1. A DNA construct comprising a transcriptional initiation region operative in plants positioned for transcription of a DNA sequence homologous to some or all of a gene encoding cellulase, said gene showing substantial homology to DNA encoded by either of the constructs TCELB6 and MCELE2.

2. A DNA construct as claimed in claim 1 comprising a transcriptional initiation region operative in plants positioned for transcription of a DNA sequence encoding RNA complementary to a substantial run of bases showing substantial homology to an mRNA encoding cellulase.

3. A DNA construct as claimed in either of claims 1 or 2 in which the DNA sequence is derived from either of the clones TCELB6 and MCELE2.

4. A DNA construct as claimed in any of claims 1 to 3 in which the DNA sequence is derived from cDNA.

5. A plant cell transformed with a DNA construct claimed in any of claims 1 to 4.

6. A genetically modified plant regenerated from a cell claimed in claim 5, or a descendant of such a plant.

7. A plant as claimed in claim 6 which shows a reduced expression of cellulase as compared with similar unmodified plants.

8. A plant as claimed in either of claims 6 or 7 which is a tomato or melon plant.

9. Fruit and seeds of plants claimed in any of claims 6 to 8.

10. The clones TCELB6 and MCELE2, and DNA constructs derived therefrom containing DNA hybridising with the cellulase-related sequences therein.

FIG.1

SEQ ID NO: 1
SEQUENCE TYPE: Nucleotide
SEQUENCE LENGTH: 223

STRANDEDNESS: Single
TOPOLOGY: linear
MOLECULE TYPE: genomic DNA

ORIGINAL SOURCE ORGANISM: Lycopersicon esculentum

FEATURES:
from 1 to 223 bp protein coding sequence

PROPERTIES: Tomato cellulase gene (part)

```
GTGGGATATG GAGCAAGGTA TCCACAGAGG ATTCATCACA GGGGATCCTC ATTACCCTCA    60
GTCGCGAACC ATCCAGCAAA GATACAATGC AGGGATGGTT TCAGTGTGAT GAACTCACAA   120
TCACCAAACC CGAACGTACT AGTAGGGGCT GTGGTAGGTG GTCCTGATGA GCATGATCGT   180
TTCCCAGACG AGCGTTCAGA TTACGAGCAA TCTGAACCTG CCA                     223
```

FIG. 1(contd.)

SEQ ID NO: 2
SEQUENCE TYPE: Nucleotide
SEQUENCE LENGTH: 223

STRANDEDNESS: Single
TOPOLOGY: linear
MOLECULE TYPE: genomic DNA

ORIGINAL SOURCE ORGANISM: Cucumis melo

FEATURES:
from 1 to 223 bp protein coding sequence

PROPERTIES: Melon cellulase gene (part)

```
GTCGGATATG GCGAGTCCTA CCCACAAAGA ATCCACCATA GAGCCACGTC GCTACCGCTG    60
ATTGCAGAGC ATCCGGCGAA GATCGACTGC TCCTCTGGCT TCTCCGCCAT GCATTCCAAT   120
TCCCCCAACC CTAATGTTCT CATCGGTGCG GTGGTTGGAG GGCCTGATCA GAATGATGGA   180
TTTCCAGATG AGCGATCGGA TTTCGAGCAA TCCGAACCAT CCA                     223
```

FIG. 2

Oligonucleotides used in PCR reactions for the generation of tomato and melon gene fragments

TCEL 10 - CCGGCCAAGATGTCATACATG

TCEL 11 - CAAGTGGGGCATTTATGTAAG

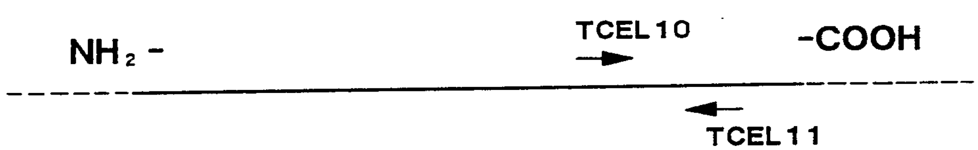
FIG. 3

Strategy for generation of Tomato and Melon cellulase gene fragments by PCR

Design oligos based on regions of homology between avocado and bean abscision cellulase sequences to specifically amplify those regions from plant genomic DNA

↓

Tomato/melon gDNA - cellulase gene



↓

PCR gDNA with oligos TCEL10 and TCEL11

SEQ ID NO: 3.
SEQUENCE TYPE: Nucleotide
SEQUENCE LENGTH: 1419

FIG. 4

STRANDEDNESS: Single
TOPOLOGY: Linear
MOLECULE TYPE: cDNA

ORIGINAL SOURCE ORGANISM: Lycopersicon esculentum

FEATURES:
from 1 to 1104 bp protein coding region

PROPERTIES: Tomato cellulase cDNA

```
GAATTCCGGG CCACAGCCCA TCCAGACACC ATTTACGTTC AGGTTGGAGA TGCAGGAAGT   60
GACCACTCTT GTTGGGAGAG ACCTGAGGAT ATGGACACCC CAAGAAGTGT GTACAAAATT  120
GACAAAAACA CTCCTGGGAC TGAAGTTGCT GCTGAAACTG CTGCTGCTCT CGCTGCTGCT  180
TCCTTAGTCT TTAGGAAATG CAACCCATCT TACTCCAAGA TACTAATCAA AAGGGCCATC  240
AGGGTGTTTG CCTTTGCTGA TAAGTATAGA GGTTCATACA GCAATGGTCT GAGAAAAGTA  300
GTGTGCCCAT ACTACTGCTC AGTTTCGGGA TATGAGGATG AGCTGTTGTG GGGTGCTGCT  360
TGGTTACATA GAGCCACAAA GAACCCAACT TATCTCAATT ATATCCAAAG GAACGGGCAA  420
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AGGGTTCTTC TTTCCAAGTC ATTTCTTGTT CAAAAGCTTC AAACTCTCCA TGATTACAAG  540
AGCCACGCAG ACAACTACAT TTGCTCCCTA ATTCCAGGCA CACCGGCTTC TCAAGCGCAA  600
TATACACCAG GAGGGCTACT CTTCAAGATG GATGATAGCA ACATGCAGTA TGTTACCTCC  660
ACTTCTTTCC TGCTAGTCAC CTATGCCAAG TACTTAACTT CTGCTCGCAT GGTTGTTAAA  720
TGTGGTGGAG TTGTTATTAC ACCAAAGAGG CTTCGAAATG TAGCCAAAAA ACAGGTGGAC  780
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GTAGGGGCTG TGGTAGGTGG TCCTGATGAG CATGATCGTT TCCCAGGCGA GAGTTCAGAT 1020
TACGAGCAAT CTGAACCTGC CACTTACATT AATGCTCCAC TTGCTGGAAC ACTCACTTAC 1080
CTTGCTCACT CATTTGGCCA ACTCTAAAGT CAAGTGTGTA GTAGAATCAA GAATGAAGCC 1140
ATGTTGGTCT TTGTTTTACT TTTCTAATTG CCTTGTTGAT CACTAGTAGT AATATAATAT 1200
ATAGTCTAAT CTAAATGGGG ATGTGCTGGT GTTGTGGTTG TATTGGCTGA GACCCTAAAA 1260
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ATTGTTAGCA GCATATTTGC CTTGTTGGAG CATATGTTGT TACTAGTATT ATTAATATTT 1380
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FIG. 5A Construction of pJRITC 1 and pJR 1MC1

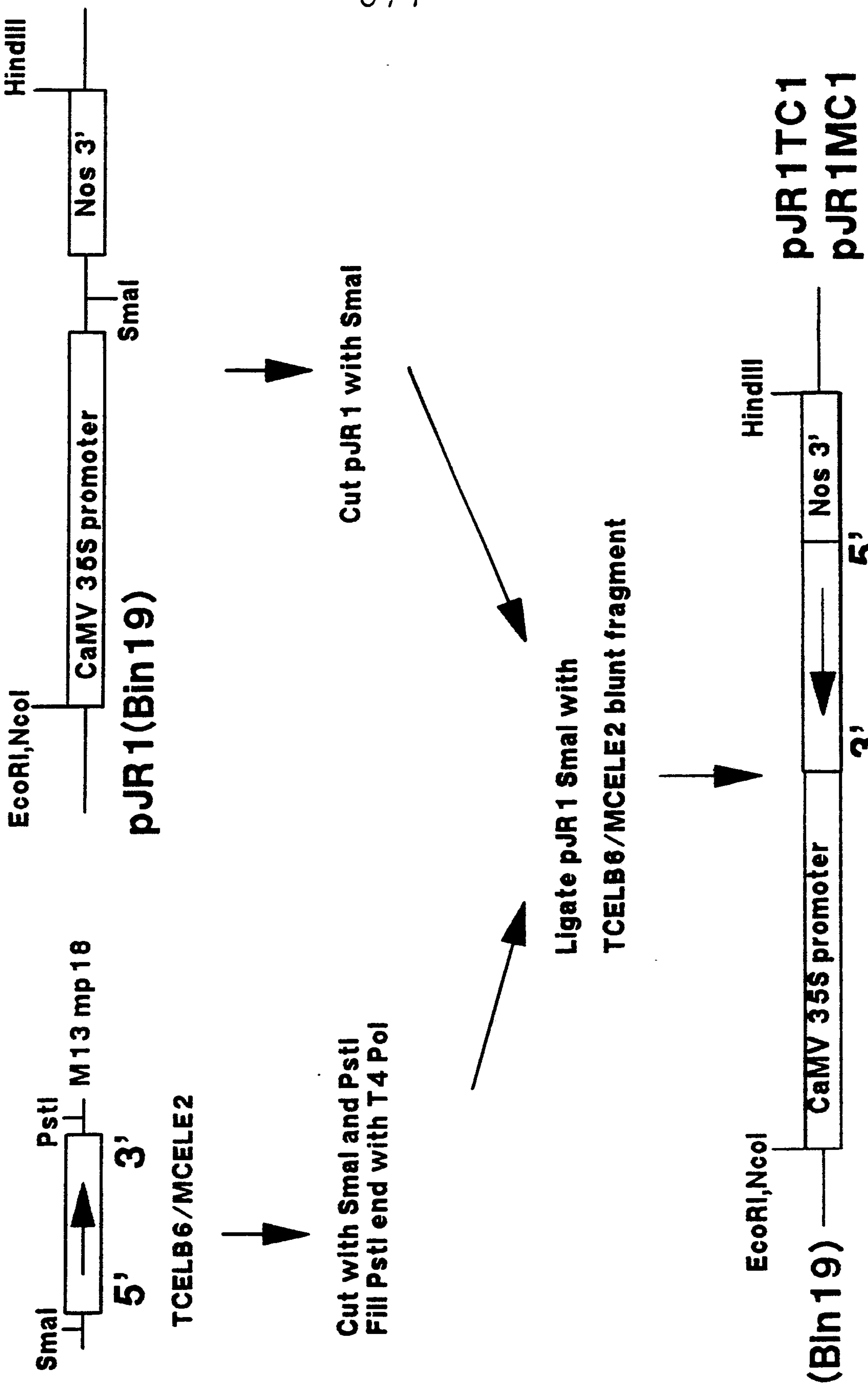
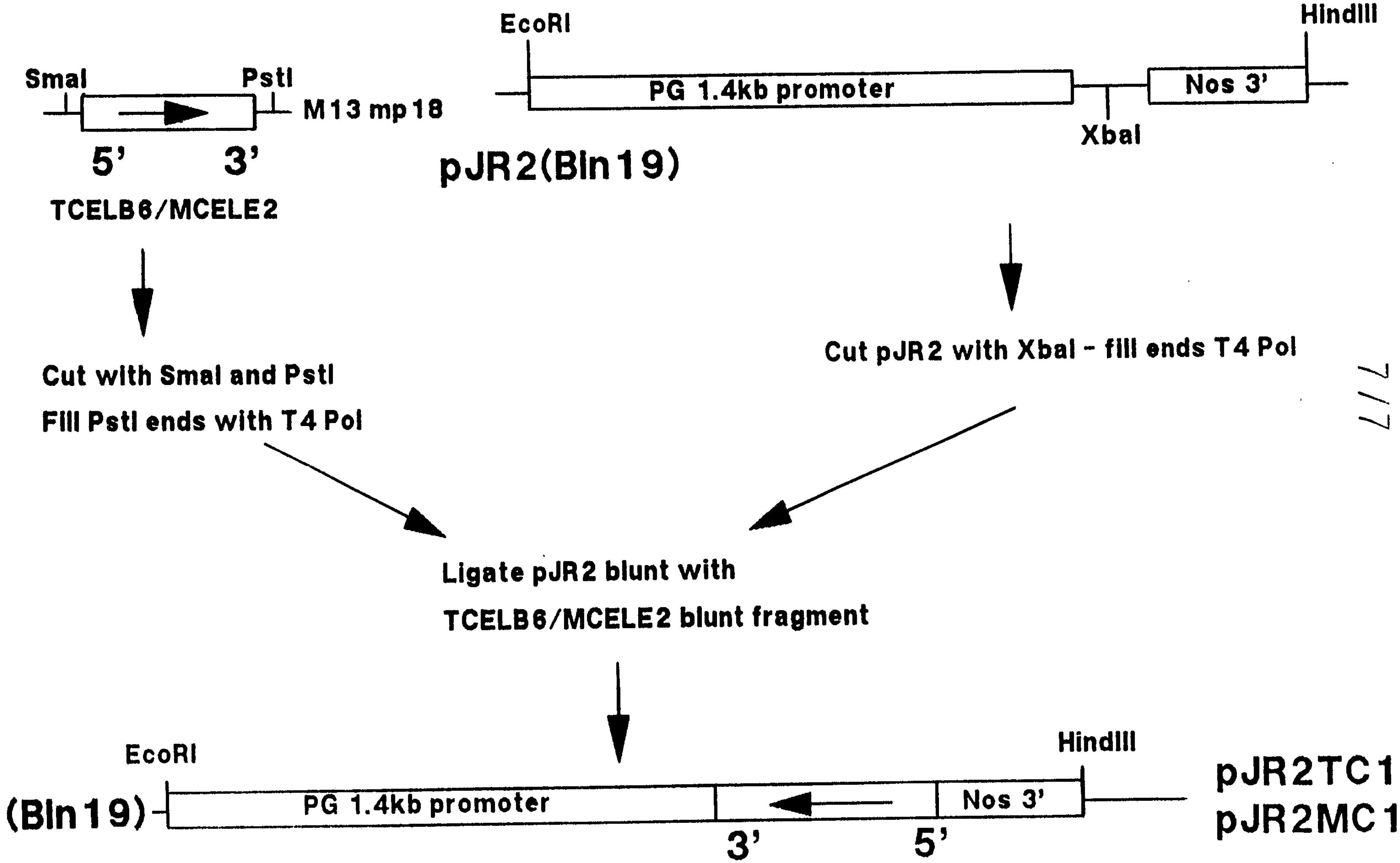


FIG. 5B Construction of pJR2TC1 and pJR2MC1



INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 91/00614

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC^5: C 12 N 15/82, 15/56, 15/11, 5/10, A 01 H 5/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC^5	C 12 N, A 01 H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Plant Molecular Biology, vol. 9, 1987 Martinus Nijhoff Publishers (Dordrecht, NL) M.L. Tucker et al.: "Avocado cellulase: nucleotide sequence of a putative full-length cDNA clone and evidence for a small gene family", pages 197-203 see the whole document	10
Y	cited in the application	1-9
	--	
Y	EP, A, 0240208 (CALGENE) 7 Ocotber 1987 see page 4, lines 29-30	1-9
	--	
X	EP, A, 0341885 (ICI) 15 November 1989 see column 2, lines 29-45; column 3, lines 20-55; claims	5-9
	--	
X	Plant Physiol, vol. 88, 1988 M.L. Tucker et al.: "Bean abscission cellulase. Characterization of a cDNA clone and regulation of gene	10
		./.

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
8th July 1991	- 9. 09. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. PEIS M. Peis

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages	Relevant to Claim No.
	expression by ethylene and auxin", pages 1257-1262, see the whole document --	
O,P, X	Plant Physiol., vol. 93, nr. 1,(suppl.) May 1990, American Society of Plant Physiologists (US) C.C. Lashbrook et al.: "Analysis of CX-cellulase gene expression in wildtype and transgenic tomato fruit", page 132, abstract no. 775 --	1-10
A	EP, A, 0271988 (ICI) 22 June 1988 see the whole document --	1-10
A	Chemical Abstracts, vol. 76, 1972 (Columbus, Ohio, US) F.E. Sobotka et al.: "Cellulase in high pigment and crimson tomato fruit", see page 240, abstract no. 56742e, & J. Amer. Soc. Hort. Sci. 1971, 96(6), 705-7 ------	2

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9100614 SA 46471

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 02/09/91
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0240208	07-10-87	AU-A- 7059787	01-10-87
		JP-A- 62296880	24-12-87
		US-A- 4801540	31-01-89
EP-A- 0341885	15-11-89	AU-A- 3469789	16-11-89
		JP-A- 2031625	01-02-90
EP-A- 0271988	22-06-88	AU-A- 7435091	11-07-91
		AU-A- 8095687	12-05-88
		JP-A- 63164892	08-07-88

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82